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14. ABSTRACT

This research program will determine whether accelerated neuron death due to increased oxidative stress resulting from mitochondrial dysfunction can be compensated or corrected by neurotrophin stimulation. The experiments will be carried out in two models of mitochondrial dysfunction. 1)hippocampal neurons from the trisomy 16 mouse, which undergo increased apoptosis and have a mitochondrial defect, that has now been identified as a decrease in Complex I-mediated respiration and 2)neurons chronically treated with the neurotoxin rotenone to induce a defect in mitochondrial function. 0.1-0.5 nM rotenone treatment has now been shown to leave hippocampal neurons vulnerable to a second oxidative stress. A unique aspect of this approach is that the neuronal responsiveness to brain derived neurotrophic factor (BDNF) will be enhanced by overexpressing the BDNF receptor via an adenovirus vector, resulting in an increase in sensitivity to BDNF. Such neurons would be expected to have an enhanced survival response to endogenous BDNF and may be more resistant to oxidative stress characteristic of Parkinson's disease and other neurodegenerative disorders.

15. SUBJECT TERMS
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Introduction

This research program will determine whether accelerated neuron death due to increased oxidative stress resulting from mitochondrial dysfunction can be compensated or corrected by neurotrophin stimulation. The experiments will be carried out in hippocampal neurons from the trisomy 16 mouse, which undergo increased apoptosis and have a mitochondrial defect, and in neurons chronically treated with the neurotoxin rotenone to induce a defect in mitochondrial function. A unique aspect of this approach is that the neuronal responsiveness to brain derived neurotrophic factor (BDNF) will be enhanced by chnaging the expression of the BDNF receptor, trkB, resulting in an increase in sensitivity to BDNF. Such neurons would be expected to have an enhanced survival response to endogenous BDNF and may be more resistant to oxidative stress characteristic of Parkinson's disease and other neurodegenerative disorders.

Overview: Year Two

In Year 1, I generated preliminary data suggesting a decrease in State 3 respiration in trisomy 16 brain supporting my hypothesis (Aim One) that there is a defect in mitochondrial function in Ts16. I have now shown that there is a small, but significant, decrease in State 3 respiration that is specific to Complex I, since Complex II-mediated respiration is unaffected. No differences in mitochondria proteins in Ts16 brain were seen by western blotting, suggesting that the defect in respiration is due to a change in function, not expression. These data will be presented at the Society for Neurosciences meeting in November, 2005 (Appendix I). Experiments with cortical mitochondria did not find a difference in calcium uptake between Ts and euploid brain cortex mitochondria, unlike the differences seen in cultured hippocampal neurons. Furthermore, while the difference in respiration is real, it's small. This could be due to the problem of looking in whole cortex to explain a defect observed for survival and calcium regulation in hippocampal neurons. To address this I have been measuring mitochondrial membrane potential in intact neurons with TMRM and have shown a marked slowing of the ability of Ts16 neuron mitochondria to repolarize after exposure to the protonionophore FCCP. With respect to the experiments proposed to study BDNF/trkB interactions with mitochondrial function in Aim II of my original project, as outlined in the previous annual report I am now planning on taking advantage of the availability of a truncated T1 trkB knockout mouse to correct the trkB defect in Ts16 and see if that corrects the mitochondrial defects. A manuscript on this mouse is currently in preparation for resubmission to Neuron (Appendix II). There have been some problems in breeding these mice and in breeding the desired number of Ts16 mice because of construction in the animal facility with both the consequent noise and the fact that the colony had to be moved twice to accommodate the construction. This has delayed some of the proposed experiments. However, because stored samples were available, some of the experiments on bcl-2 and bax expression proposed for Year 3 were moved up to this year. The mouse colonies are now in their permanent new headquarters and breeding productivity is back up, so there are no problems anticipated for the next two years.

Research Accomplishments:

Oxygen consumption in Ts16 brain.

It has been suggested that there is a complex I defect in Ts16 mitochondria (Schuchmann and Heinemann, 2000a) on the basis of differing effects of the complex I inhibitor, rotenone, on free radical generation in euploid and Ts16 neurons. However, these results are difficult to interpret, since they showed an inhibition of ROS by rotenone, in contrast to the increase more commonly seen when rotenone prevents complex I from passing electrons on down the electron transport chain (ETC) (see e.g. Sherer et al, 2001; 2002). It is possible that these results reflect artifactual changes in hydroethidine fluorescence. Thus, the question of whether there is an ETC defect in Ts16 remains unanswered. ETC defects in Ts16 could lead to increased OS and decreased energy production and predispose Ts16 neurons to die. Electron transport chain activity can be measured using an oxygen electrode with isolated mitochondria, cells permeabilized with digitonin, or tissue homogenates (Fiskum et al., 2000). Neuron yields from E16 hippocampal preparations are typically 400,000 cells/brain. Since on the order of 10^7 cells are needed for oxygen consumption measurements in permeabilized cells, we cannot do these experiments in purified hippocampal cultures. I initially proposed that it is probable that the Ts16 defect is present in all neurons. In Year I, I measured respiration in crude homogenates from embryonic cortex and the results suggested a decrease in oxygen consumption in Ts16. Several technical improvements were made in Year I to improve my ability to measure oxygen consumption in the small amounts of tissue from the embryonic brain, principally using crude mitochondrial fractions instead of homogenates and a mini-chamber from Strathkelvin.

Mitochondria were prepared by homogenization of E16 cortex on ice in KCl medium with 0.5 mM EDTA. The homogenate was centrifuged once at a low speed to remove unhomogenized material and then at a high speed to separate the homogenate into cytosolic (supernatant) and mitochondrial (pellet, this fraction can contain other intracellular organelles) fractions. Protein was determined using the micro-Lowry method and the mitochondrial fraction was used unless otherwise indicated. The maximal rate of coupled State 3 respiration through Complex I was determined in the presence of the NAD-linked substrates glutamate and malate. Then ATP synthesis was inhibited with oligomycin to measure resting (State 4) respiration, which reflects the rate of leakage of protons back across in inner mitochondrial membrane. For comparison respiration was measured in the presence of the Complex I inhibitor rotenone and with the FAD-linked substrate succinate to measure State 3 respiration through Complex II. I have now shown (Figure I) that there is a 15% decrease in State 3 respiration in Ts16 cortex that is specific to Complex I. This is exciting because Complex I has been implicated in Parkinson's disease. Both Ts16 and euploid mitochondria appeared to be equally well coupled (no significant differences in the State 3:State 4 ratio were found). Although the magnitude of the defect in Ts16 respiration is very small, small differences may have cumulative effects that are important in chronic diseases.

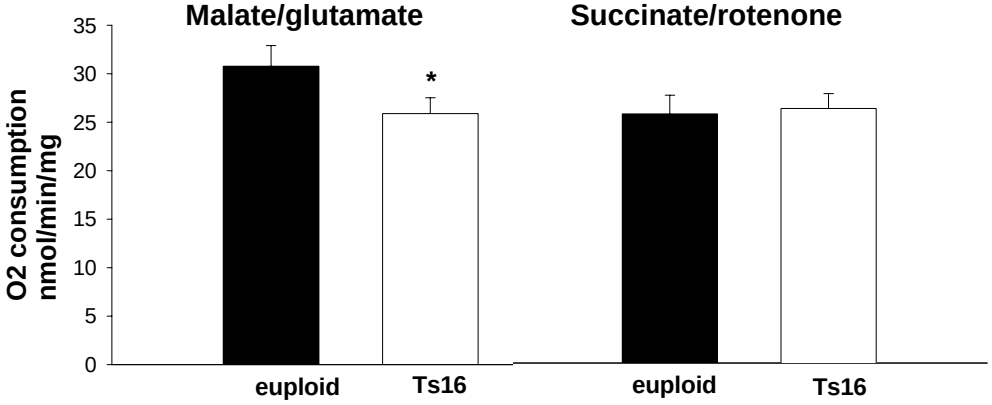

Figure 1: Selective impairment of Complex I-mediated State 3 respiration in Ts16. Oxygen consumption was measured in crude mitochondrial fractions from embryonic day 16 brain using the minichamber and a Clarke-type electrode from Strathkelvin. Data are means and SEM for 4-6 preparations. * significantly different from euploid, $p<0.05$.

Mitochondrial composition in Ts16

To determine if the decrease in Complex I-mediated respiration could reflect a decrease in Complex I, I used the cocktail of anti-electron chain antibodies from MitoSciences. Figure 2A shows the results for three different preparations of euploid and Ts16 brain. No differences were observed by inspection, a finding confirmed by densitometry, Figure 2B. Therefore, the defect in Ts16 respiration may reflect changes in protein function rather than protein expression.

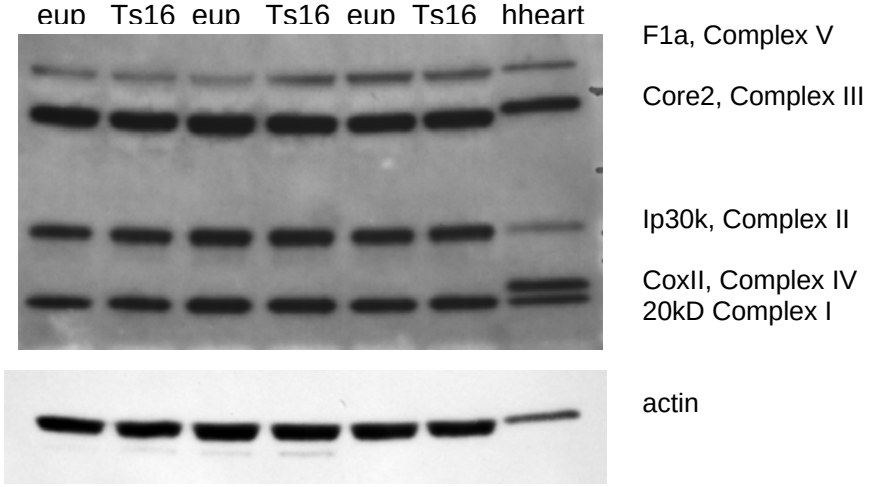

Figure 2A: Mitochondrial proteins in euploid and Ts16 cortex. Mitochondrial fractions from embryonic day 16 mouse brain were isolated and probed with the OXPHOS cocktail from Mitosciences, stripped and reprobed for actin. The legend on the right indicates the protein detected and its electron transport chain complex. Note that the Complex IV antibody only detects Cox II in the human heart postive control from Mitocsciences, not in mouse or rat (not shown).

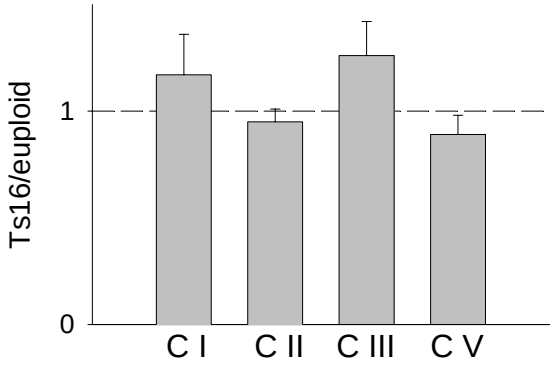

Figure 2B: Mitochondrial proteins in Ts16 brain cortex mitochondria at embryonic day 16. Data from Oxphos blots (Figure 2A) were scanned, band density was normalized to the actin band and expressed as a fraction of the euploid band from the same preparation. Data are means and SEM for 5 preparations.

Calcium uptake by Ts16 mitochondria

In hippocampal neuron cultures I had previously shown that Ts16 cells had impaired calcium regulation and neuroblastoma cells with chronic Complex I deficiencies have been reported to have altered calcium signaling (Sherer et al., 2001). In order to determine if the Ts16 calcium defect is present at the level of the mitochondria, calcium uptake was measured in a spectrofluorimeter using the isolated mitochondrial fraction (described above) with fura-6FF as the calcium indicator. As shown in Figure 3A, repeated pulses of calcium (20 nmoles/addition) are added at 5 minute intervals. The solution calcium initially rises and then the mitochondria take up the calcium and solution calcium returns to baseline. With repeated pulses, the mitochondria eventually fail to take up further calcium. The cumulative calcium to the last completely taken up addition is calculated as the calcium capacity and expressed per mg protein of the mitochondrial fraction. Figure 3B shows that there was no significant difference in the capacities of Ts16 and euploid mitochondria, although both showed a small increase in the presence of cyclosporin A.

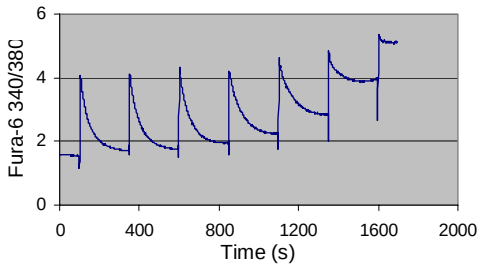

Figure 3A: Calcium uptake in euploid cortical mitochondria. An example of an experiment with 0.2 mg euploid cortical mitochondria, 20 nmoles calcium/addition.

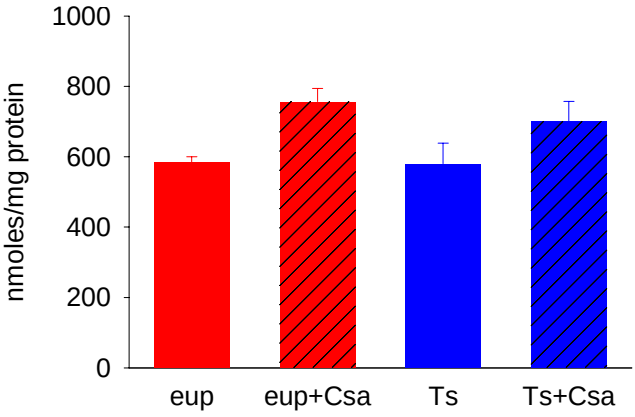

Figure 3B: Calcium uptake by cortical mitochondria. Maximal calcium uptake by corltical mitochondrial from euploid (red) and Ts16 (blue) embryonic day 16 brain in the absence (solid bar) or presence (hatched bar) of 2 uM cyclosporin A (Csa). Data are means and SEM of 3-6 experiments.

The next set of experiments planned for the cortical mitochondria is to measure free radical production using the Amplex red assay. Inhibition of respiration at Complex I could lead to generation of increased free radicals. As a control to see if the assay is sensitive enough to detect the effects of a 15% inhibition in Complex I, I have done dose-response curves with added rotenone measuring respiration and determined that 1-10 nM rotenone provides approximately a 15% inhibition of malate-glutamate-mediated euploid mitochondrial respiration (Figure 4). I plan to measure free radical generation in euploid, Ts16 and rotenone-treated euploid mitochondria.

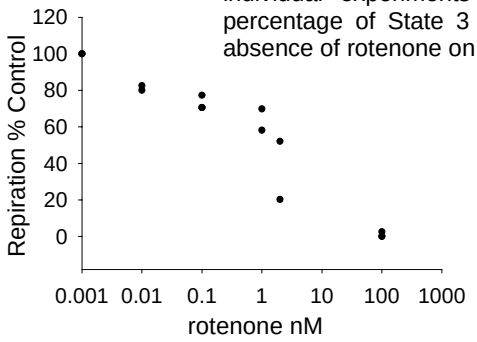

Figure 4: Rotenone inhibition of State 3 respiration. Oxygen consumption was measured as in Figure 1 with malate/glutamate as substrates in the presence of different concentrations of rotenone. Data are the values for individual experiments expressed as a percentage of State 3 respiration in the absence of rotenone on the same day.

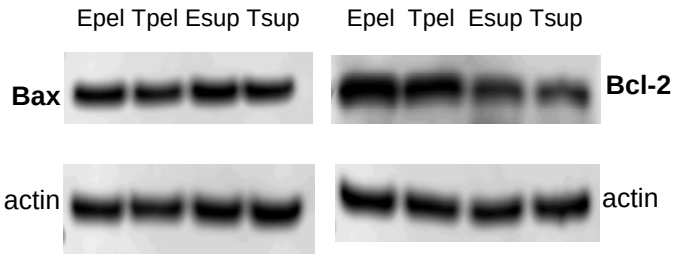

Figure 5A: Bcl-2 and bax in euploid and Ts16 cortex. Mitochondrial (pel) and cyotsolic (sup) fractions were isolated from euploid and Ts16 embryonic day 16 cortex, probed with antibodies to bax (Upstate Bioscience) or bcl-2 (Santa Cruz) and the blots reprobed for actin.

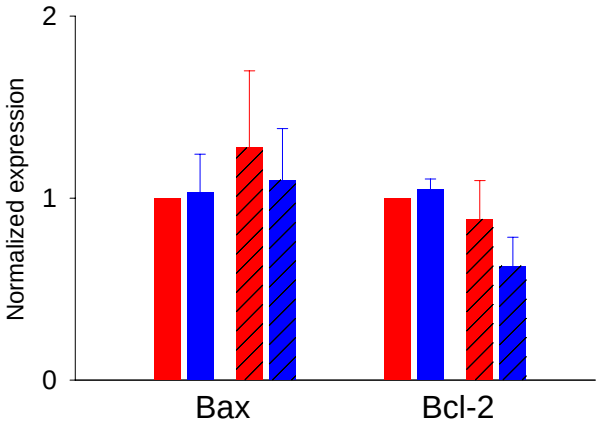

Figure 5B: Bax and Bcl-2 expression in euploid and Ts16 embryonic day 16 cortex. Data were normalized to actin and expressed relative to the euploid mitochondrial fraction from the same preparation. Data are means and SEM from 3 independent preparations for euploid (red) and Ts16 (blue) mitochondrial (solid bar) and cytosolic (hatched bar) fractions.

Bcl-2 and Bax expression

For the bcl-2 family proteins, both their total amount in the cell and their distribution between the mitochondria and the cytoplasm affect their function (Hsu et al., 1997; Polster et al., 2001; Yang et al., 1997; zha et al., 1999). I plan to measure the expression of bax, bcl-2, bad and bid in mitochondrial and cytoplasmic fractions from brain and cultured cells. In initial experiments, the supernatant (cytosolic fraction) and the pellet (mitochondrial fraction) from the isolation of mitochondria from embryronic brain (see above) were fractionated by SDS-PAGE, the proteins were transferred to nitrocelluose membranes and analyzed by Western blotting for the pro-apoptotic bax and the anti-apoptotic bcl-2 as shown in Figure 5. Both Bax and Bcl-2 were readily detected in the cytosolic and mitochondrial fractions, although there was no difference between euploid and Ts16 brains. This approach will now be used on the hippocampal neuron cultures.

Mitochondrial function in hippocampal neurons

The small decrease in Complex I-mediated respiration and the lack of defect in mitochondrial calcium uptake in Ts16 cortex may suggest that the defect(s) seen in hippocampal neurons in vitro are not (as) present in the developing embryonic cortex. So I wanted to look at mitochondrial function in cultured neurons using a method that did not involve a calcium challenge- because Ts16 neurons could have calcium homeostasis due to non-mitochondrial changes and measuring mitochondrial function in response to a calcium challenge could give misleading results. I have developed a method of using a brief exposure to the proton ionophore FCCP (Nicholls and Ward, 2000; Virtual Cell simulation, www.buckinstitute.org/Nicholls). This uncoupler depolarized the mitochondrial membrane (with little effect on plasma membrane potential). Recovery of the mitochondrial membrane potential will be due to a combination of washout of the FCCP and proton pumping by the electron transport chain. Using the membane potential dye TMRM in a low (unquenching) concentration, a short pulse of FCCP leads to a rapid drop followed by a slow recovery of fluoresecence intensity (535 excitation, 590 emission).Figure 6A shows a representative experiment when cultures are imaged (Nikon Eclipse inverted microscope, Hamamatsu Orca ER camera, Universial Imaging data analysis) and the change in the whole cell signal expressed as the change in intensity from initial fluorescence divided by the initial fluorescence (DF/F) to normalize signals from different cells. It can be seen (Figure 6B) that the recovery of the TMRM signal is linear through its middle portion, although , in reality, this recovey contains at least two components- washout of FCCP and proton pumping. Assuming that there are no major differences in FCCP washout between euploid and Ts16 neurons, the rate of recovery of the TMRM signal may be taken to reflect differences in the rate of proton pumping between the cells. Figure 6C shows the results of three separate experiments where there is a

significant slowing of the Ts16 recovery rate. This may represent a quantitative measure of differences in Ts16 and euploid mitochondrial function. Experiments in progress to validate this method of measuring mitochondrial function include using NAD(P)H fluorescence, rather than TMRM to follow mitochondrial recovery from the brief FCCP exposure and using other drugs (KCN and oligomycin) to drop the mitochondrial membrane potential.

Manipulation of Trk B expression in euploid and Ts16

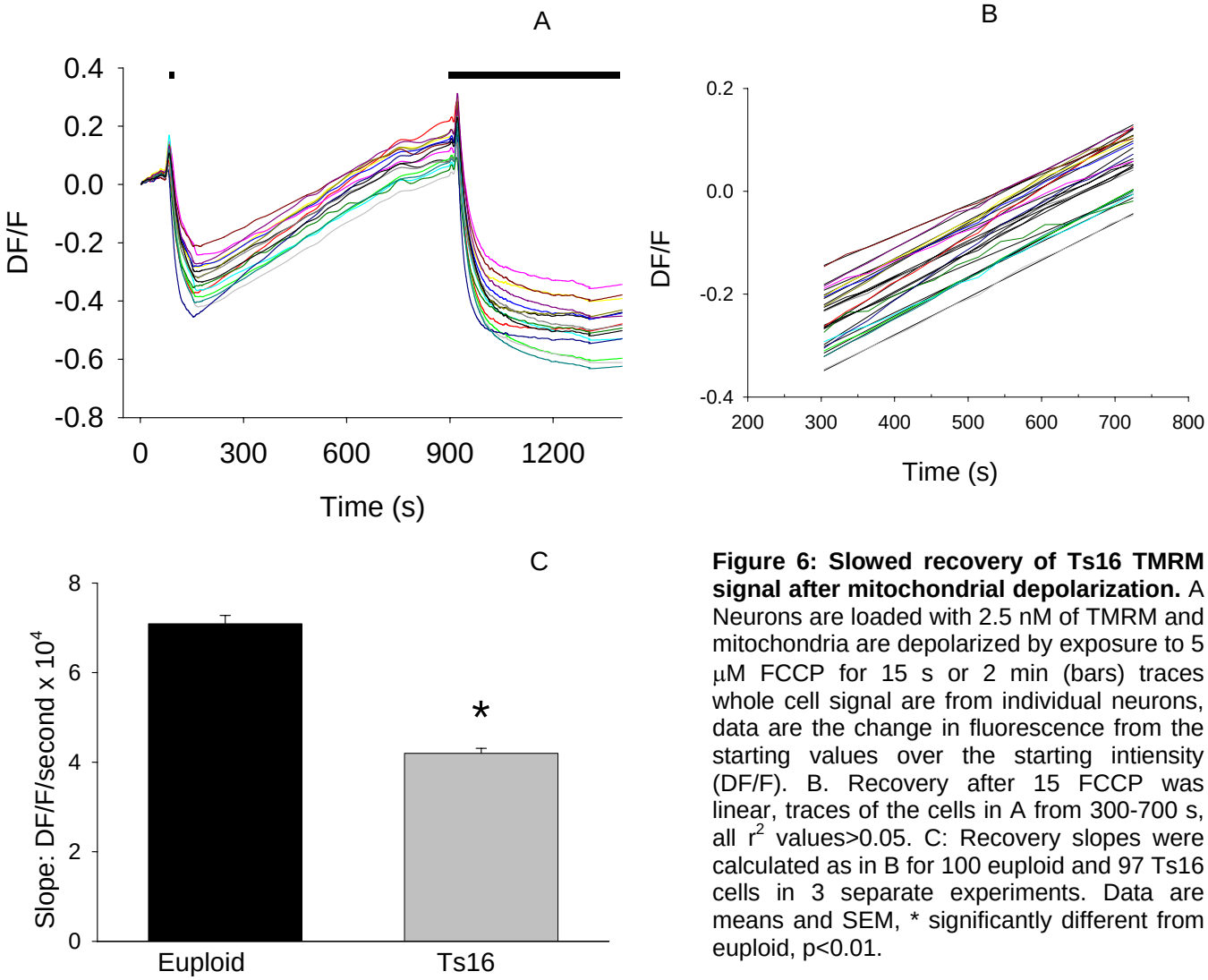

Figure 6: Slowed recovery of Ts16 TMRM signal after mitochondrial depolarization. A Neurons are loaded with 2.5 nM of TMRM and mitochondria are depolarized by exposure to 5 μM FCCP for 15 s or 2 min (bars) traces whole cell signal are from individual neurons, data are the change in fluorescence from the starting values over the starting intiensity (DF/F). B. Recovery after 15 FCCP was linear, traces of the cells in A from 300-700 s, all r^2 values>0.05. C: Recovery slopes were calculated as in B for 100 euploid and 97 Ts16 cells in 3 separate experiments. Data are means and SEM, * significantly different from euploid, $p<0.01$.

One of the questions to be addressed in this project was the extent to which neurotrophins and neurotrophin receptor activation can correct mitochondrial defects. In our earlier work (Dorsey et al., 2002) we had shown that Ts16 neurons have defective BDNF signaling because of an over expression of the truncated form of the BDNF receptor trkB (which acts as a dominant negative repressor). In my previous annual report I reported that Dr. Susan Dorsey has now developed a truncated trkB knockout mouse. In more recent experiments Ts16/truncated trkB-negative mice have been generated. Neuron survival appears to be normal in these mice (Appendix II). I am now proceeding to initiate studies on hippocampal neurons from these mice using the approaches described in my initial application and above to determine whether Ts16 mice have a mitochondrial defect that is separate from their defective trk B signaling or if correcting the trk B defect can correct Ts16 function- a finding with important implications for neurotrophin therapies.

Key Research Accomplishments:

- Respiration by mitochondria from embryonic Ts16 cortex shows a small but significant decrease that is specific to Complex I. This decrease is not accompanied by changes in mitochondrial protein expression of calcium uptake capacity.
- Using a fluorescence imaging method to quantify mitochondrial function in living cells revealed that Ts16 neurons have a two-fold slowing of their ability to recover mitochondrial membrane potential after challenge with a proton ionophore. This method will be used in future studies.
- Truncated trkB knockout mice have now been bred to generate Ts16 mice with reduced expression of the truncated trkB. Neurons from these mice are available for experiments to study the links between neurotrophins and mitochondrial function.

Conclusions:

One conclusion of the past year's work is that Ts16 cortex is not the best material to use for studying defects in Ts16 mitochondrial function, this possibility was explored in the initial grant application. I had hoped that the Ts16 defect would be easily detectable in cortex in order to do experiments requiring (relatively) large amounts of tissue. I am currently preparing the Ts16 cortex work to date for publication and the majority of the future work will now be concentrated on studies of hippocampal neurons. In this light, the experiments on the rates of recovery of TMRM fluorescence after FCCP depolarization show quantitative differences between euploid and Ts16 neurons. Experiments in the coming year will determine the mechanism of this difference and the degree to which changing the neurotrophin signaling pathways in the cells with the truncated trkB knockout mouse will correct the mitochondrial dysfunction.

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Appendix One

MITOCHONDRIAL FUNCTION IN THE TRISOMY 16 MOUSE CORTEX

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Increased intracellular calcium and free radical generation have been reported in mouse trisomy 16 (Ts16) neurons in culture and may be linked to the accelerated death of these cells (Bambrick and Krueger,1999, Schuchmann and Heinemann, 2000). To determine whether there is a mitochondrial defect in Ts16 that could underlie these results, we assessed several measures of mitochondrial function in cortex of Ts16 and control (euploid) mouse brain at embryonic day 17. Cortices were isolated and a crude mitochondrial fraction prepared and resuspended in KCl buffer. To assess mitochondrial electron transport chain activity, oxygen consumption was measured using a Clarke-type electrode in KCl buffer with ADP. Ts16 brain mitochondria had a selective 15 % reduction in Complex I-mediated respiration ($p<0.01$) in the presence of the NADH-linked substrates malate/glutamate, with no defect in FADH-linked Complex II-mediated respiration, measured in the presence of rotenone/succinate. Western blot analysis of euploid and Ts16 brain mitochondria did not show any quantitative differences in several mitochondrial proteins, including the 39 and 20 kDa subunits of Complex I. To determine if the decreased Complex I-mediated respiration had functional consequences for the Ts16 mitochondria, the generation of reactive oxygen species was measured using an Amplex Red assay for hydrogen peroxide. Rates of hydrogen peroxide generation were similar in the two brains. Altered calcium metabolism by Ts16 mitochondria has been proposed as a mechanism for the altered calcium responses found in Ts16 neurons. When calcium uptake was measured in the presence of malate/glutamate and ATP, using fura-6FF, the uptake capacities of Ts16 and euploid mitochondria were found to be similar. The present results suggest that there is a Complex I electron transport chain defect in trisomy 16 cortex, but that it may not be linked to altered free radical generation or calcium uptake.

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In vivo restoration of physiological levels of truncated TrkB receptor rescues accelerated neuronal cell death in the trisomy 16 mouse

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Running Head: Truncated trkB causes neuron cell death

Key Words: trkB; BDNF; gene targeting; knockout mouse; neurodegeneration; trisomy 16,

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Abstract.

Imbalances in neurotrophins or their high affinity Trk receptors have long been reported in neurodegenerative diseases. However, a molecular link between these molecules and neuronal cell death has not been established. We have previously shown that in the trisomy 16 (Ts16) mouse model hippocampal neurons undergo accelerated cell death that cannot be rescued by administration of brain derived neurotrophin factor (BDNF). Ts16 neurons have normal levels of the TrkB tyrosine kinase receptor but an up-regulation of the TrkBT1 truncated receptor isoform. Here we show that restoration of the physiological level of the TrkBT1 receptor by gene targeting rescues Ts16 neuronal death. Moreover, it corrects resting Ca^{2+} levels and the TrkB full-length responsiveness to BDNF of Ts16 hippocampal neurons. These data provide a direct link between neuronal cell death and abnormalities in Trk neurotrophin receptor levels.

Introduction.

Linking specific genes to developmental or pathological conditions is one of the most challenging tasks in experimental biology. While gene targeting can identify a direct involvement of a gene in specific development processes, in pathological conditions, this task has been much more complex due to the numerous alterations in gene or protein expression levels that are associated with the disease. Most studies are correlative and rarely can an unambiguous connection between a gene product and a phenotype be established. For example, numerous lines of evidence support the notion that Huntington's disease is caused by a mutation in huntingtin, however, a causal relationship between mutant protein expression and progression of the disease is not clear. This relation between mutant protein expression and disease progression was later shown with a conditional mouse model where the blockade of expression of the mutant protein led to the disappearance of inclusions and the amelioration of the behavioral phenotype (Yamamoto et al. 2000). Neurotrophins and their receptors are critical for normal nervous system development (reviews). Changes in their expression have been reported in a variety of pathological conditions, including neurodegenerative diseases (). However, while they have been considered targets for clinical intervention, their direct role in pathologies of the nervous system is still unclear. Heterozygous mice with targeted deletion of neurotrophin genes have provided a useful system to investigate the effects of reducing neurotrophin levels in the adult, which include obesity and increases aggression in $BDNF^{+/-}$ mice, and memory deficits in $NGF^{+/-}$ mice. (Kernie, Lyons, NGF+/-, Linnarson etc). However, to date, there is no information on whether dysregulation of Trk receptor isoforms, often observed in pathological conditions, may affect neuronal physiology. Neurons from the trisomy 16 (Ts16) mouse represent a useful model to study the effects of dysregulation of a variety of genes and its relative impact on neuronal survival. Ex vivo, hippocampal or cortical neurons undergo accelerated cell death that can be rescued by a variety of exogenous factors including fibroblast growth factor 2 and neurotrophin-3 (). However, in these rescue experiments it is difficult to identify the primary defects leading to cell death because pharmacological activation of strong pro-survival pathways could mask the intrinsic cellular defect.

We previously reported that the accelerated death of Ts16 neurons could not be prevented by the exogenous application of brain-derived neurotrophic factor (BDNF) (Dorsey, et al., 2002),

a potent autocrine survival factor for hippocampal neurons. Although Ts16 neurons expressed the full-length, catalytically-active isoform of the BDNF receptor, trkB, they over-expressed the T1 isoform of truncated trkB (TrkB.T1), which can act as a dominant-negative inhibitor of BDNF signaling (Eide et al., 1999). Consistent with this mechanism, the premature neuron death was rescued by adenovirus-mediated overexpression of full-length TrkB, suggesting that BDNF signaling requires an excess of full-length trkB over TrkB.T1expression. Reducing expression of TrkB.T1 would be predicted to similarly restore the dominance of full-length trkB and consequently restore BDNF signaling.

In this study, we used a gene targeting approach to reduce TrkB.T1 to physiological levels in the Ts16 mouse in order to test the hypothesis that overexpression of TrkB.T1 is the primary cause of the accelerated Ts16 hippocampal neuron death. We found that genetic reduction of TrkB.T1 expression to a normal level enhances trkB activation and rescues neuron cell death. Our results indicate that a minimal level of neurotrophin signaling levels is required for neuron survival and suggest that overexpression of truncated trk isoforms can reduce signal strength to below this minimal level. Small alterations in neurotrophin/Trk receptor activation like those seen in mouse Ts16 may be directly linked to neurodegenerative diseases.

RESULTS

Selective deletion TrkB.T1 isoform.

To decrease the levels of TrkB.T1, we first used a gene targeting approach remove the *TrkB.T1* coding exon, which is located between the juxtamembrane and the tyrosine kinase coding exons (Fig. 1a). To avoid disruption of the full-length TrkB isoform containing the tyrosine kinase, we inserted loxP sites into the genomic regions that showed no conservation between the mouse and human sequence (Fig. 1b). We reasoned that a lack of conservation between the two mammalian species indicated that these areas are not important for splicing regulation. Recombinant embryonic stem cell clones and subsequently, mice containing the correctly targeted *TrkB.T1* exon, were obtained as described in Methods and Fig. 1 (b, c). The absence of TrkB.T1 in mutant mice was verified in whole brain lysates (Fig. 1d). Since a TrkB-specific antibody raised against the extracellular domain showed the presence of a background band at about the same position on the gel as TrkB.T1, we prepared whole brain wheat-germ lysates and probed with the same antibody. This procedure, which was used to enrich for glycosylated proteins such as Trk receptors, eliminated the background band (Fig. 1d right panel). We next investigated whether elimination of the genomic sequence between the transmembrane and tyrosine kinase coding exons would affect the expression of the full-length TrkB receptor (Fig. 1d). No difference in the level of full-length TrkB, normalized to actin expression in the same samples, was found between the TrkB.T1 mutant and control mice, suggesting that targeting of the TrkB.T1 truncated isoform did not affect the expression level of full-length TrkB (data not shown). To investigate whether splicing of the different TrkB isoforms was affected by the targeting, we analyzed isoform expression in cultured primary astrocytes, a cell type that expresses only truncated TrkB (Rose et al. 2003). Neither full-length TrkB nor TrkB.T1 expression was noted in astrocytes from homozygous *TrkB.T1* mutant mice, suggesting that the genomic elements controlling splicing were not affected. Moreover, we observed an approximately 50% reduction of the TrkB.T1 receptor level in the heterozygous mutant astrocytes, suggesting a potentially useful strategy to correct TrkB.T1 levels in the Ts16 mouse (Fig. 1e).

In vivo reduction of TrkB.T1 restores Ts16 hippocampal neuron survival.

To determine whether reducing TrkB.T1 levels would rescue Ts16 hippocampal neurons from premature death, we intercrossed *TrkB.T1* homozygous mice with the Robertsonian Ts16 parental strain, which, is doubly heterozygous for chromosomal translocations 6:16 and 17:16 (Gearhart, et al., 1986). The resulting progeny were all heterozygous for *trkB.T1* and were either euploid or trisomic (monosomic conceptuses are early embryonic lethal). Cultured hippocampal neurons were prepared from littermate euploid and trisomic embryos at day 15.5 (E15.5) and maintained in serum-free medium containing the chemically defined supplement B27 (Brewer, et al., 1993). By the second day *in vitro*, the cultures consisted primarily of post-mitotic, differentiated neurons with extensive process formation, as previously described (Dorsey et al., 2002). At three days *in vitro*, we examined the levels of TrkB.T1 in hippocampal neurons from the Ts16 mice heterozygous for *trkB.T1* and found that TrkB.T1 protein had been reduced to levels indistinguishable from wildtype (Figure 2a). Next, we assayed the survival of the Ts16; *TrkB.T1*$^{+/-}$ hippocampal neurons *in vitro*. We cultured hippocampal neurons on gridded glass coverslips (Bambrick et al., 1995) and followed the survival of individual neurons over seven days *in vitro* (Dorsey et al., 2002). Consistent with our previous reports (Bambrick et al., 1995; Dorsey et al., 2002), about 15% of wildtype, euploid neurons and 50% of Ts16 neurons died within seven days (Fig. 2b). In contrast, the survival of Ts16 neurons heterozygous for *trkB.T1* was indistinguishable from wildtype euploid neuron survival, indicating a complete rescue of the premature neuronal death phenotype (Fig. 2b). Furthermore, at day six *in vitro*, in contrast to Ts16 *TrkB.T1*$^{+/+}$ neuron cultures, which exhibited widespread neurite fragmentation and cellular debris, neuron cultures from Ts16 *TrkB.T1*$^{+/-}$ fetuses had complex, non-fragmented neurites and little or no cellular debris similar to wildtype euploid neurons (Fig. 2c). These findings suggest that restoring physiological expression levels of a single gene isoform, TrkB.T1, is sufficient to rescue the premature hippocampal neuronal cell death phenotype of Ts16 neurons.

TrkB response to BDNF is restored in Ts16 neurons by in vivo reduction of TrkB.T1.

To examine the mechanism underlying the restoration of Ts16 hippocampal neuron survival, we analyzed the BDNF-induced phosphorylation of full-length TrkB. As previously reported, in Ts16 neurons TrkB phosphorylation was decreased to about 60% of that of euploid neurons, suggesting that the increased truncated TrkB inhibits TrkB signaling (Fig. 3a; Dorsey

2002). However, when we reduced the levels of TrkB.T1 *in vivo* by breeding Ts16-*TrkB.T1*$^{+/-}$ fetuses, the BDNF-induced phosphorylation of TrkB was restored to 85-90% of wildtype euploid levels (Figure 3a, b). These data support the notion that the over-expression of TrkB.T1 in Ts16 neurons acts to inhibit full-length TrkB signal transduction, which, in turn, causes premature neuron death and the inability to fully respond to BDNF.

Intracellular cytoplasmic Ca^{2+} levels are restored in Ts16 *TrkB T1*$^{+/-}$ neurons.

Ts16 neurons and astrocytes have increased levels of resting intracellular Ca^{2+} (Bambrick, et al., 1997; Muller, et al., 1997; Schuchmann, et al., 1998). Recently, it was shown that BDNF induces an increase in cytoplasmic Ca^{2+} in astroglia. Because cultured glial cells do not express measurable levels of full-length TrkB, this response is mediated by the truncated TrkB.T1 receptor, although a pathophysiological link between the TrkB.T1 and Ca^{2+} levels is unknown. Therefore, we investigated whether restoring TrkB.T1 to physiological levels could reduce resting Ca^{2+} levels in Ts16 neurons. In agreement with previous reports, we found a small, but significant, increase in resting intracellular Ca^{2+} levels in Ts16 hippocampal neurons ($Q=2.424$, $p < 0.05$) between four and seven days *in vitro* (Figure 3c). Interestingly, when TrkB.T1 expression was reduced to euploid levels by breeding Ts16-*TrkB.T1*$^{+/-}$ mice, we found a significant decrease ($Q=5.484$, $p < 0.05$) in intracellular resting level of cytoplasmic Ca^{2+} (Figure 3c). These data suggest that the altered Ca^{2+} homeostasis observed in Ts16 hippocampal neurons is a consequence of TrkB.T1 dysregulation.

Discussion

In this study, we demonstrate that correcting truncated TrkB.T1 receptor expression to physiological levels is sufficient to rescue the accelerated death of Ts16 neurons. In turn, rescued neurons show both an increase in the phosphorylation level of the TrkB tyrosine kinase receptor in response to BDNF and a restoration of intracellular Ca^{2+} levels.

Alterations in neurotrophins or their Trk receptor levels have been reported in a variety of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and Alzheimer's, Huntington's and Parkinson's diseases (reviewed in Dawbarn 2003). However, it is still unclear whether changes in expression of these receptors are involved in the pathogenic process or are an

indirect effect of the disease. A major problem with testing this hypothesis has been the limited number of suitable models. Ts16 hippocampal neurons provide a tool to study genetic abnormalities underlying neuronal cell-death. The cause of the accelerated cell death has the potential to be multigenic since hundreds of genes are dysregulated in trisomies (Mao et al. 2003). Surprisingly, we have found that an alteration in TrkB receptor signaling is sufficient for the development of this phenotype, suggesting that dysregulation of a single gene is sufficient to cause cellular alterations resulting in neuron death. It should be noted that since the *TrkB* gene is located on mouse chromosome 13 (and on human chromosome 9) and consequently not triplicated in Ts16, the altered expression of TrkB isoforms observed in Ts16 neurons must be secondary to the increased gene dosage of one or more mouse chromosome 16 genes.

Expression of TrkB receptor isoforms is dynamically, yet tightly, regulated during development of the mammalian or chicken nervous system, suggesting a critical role for this receptor. Expression of the full-length TrkB precedes that of the truncated isoforms (Escandon, Allendoerfer). Interestingly, in the mammalian cortex, the shift to a predominance of truncated TrkB occurs at times that correlate with the onset of cell death and maturation of axonal connections (Allendoerfer, ohira k 1999). Thus, imbalances in TrkB receptor isoform levels in pathological conditions may lead to cell death with a mechanism similar to that used during the critical period of cell death and axonal remodeling associated with the nervous system developmental plasticity.

We previously reported a selective failure of BDNF to support the survival of Ts16 hippocampal neurons due to the elevated expression of TrkB.T1 relative to full-length TrkB and that this defect could be corrected by overexpressing full-length TrkB (Dorsey et al., 2002). Now we report that reducing the expression of TrkB.T1 to its level in euploid neurons can restore BDNF-induced survival of Ts16 neurons. These data suggest that neurodegeneration may not be the result of a diminished supply of neurotrophins and provide direct evidence that neurons must express the correct set of receptor isoforms to transduce a proper survival signal in response to neurotrophins.

Interestingly, overexpression of truncated trkB has also been reported in the hippocampus of human Alzheimer's disease patients (Ferrer, et al., 1999), suggesting a similar mechanism may participate in hippocampal neuron degeneration in this disease. Moreover, in ALS patients BDNF mRNA and protein are dramatically up regulated in muscle as well as total TrkB mRNA

in the spinal cord (Kust 2002; Mutoh et al., (2000). Yet, phosphorylation of the TrkB receptor is reduced (Mutoh et al., 2000). Thus, it is possible that as we demonstrate in Ts16 neurons, TrkB signaling impairments in ALS are not at the level of neurotrophin supply but rather at the level of the TrkB receptor isoforms being expressed. Determination of truncated and full-length TrkB receptors ratios should help in identifying whether ALS and Ts16 neurons have similar deficits. and may help to explain why therapeutic use of BDNF has been unsuccessful in ALS clinical trials ().

We have found that, in Ts16 hippocampal neurons, restoration of physiological levels of TrkB.T1 not only restores BDNF-mediated survival, but also corrects resting Ca^{2+} levels. While it was known that truncated TrkB alone mediates the regulation of intracellular Ca^{2+} levels by BDNF in astrocytes, the function of TrkB.T1 in neurons, which express both full-length and truncated TrkB isoforms, is more complex. Our data suggest that, in neurons, proper TrkB.T1 levels (or the relative proportion of full-length TrkB to TrkB.T1) are critical for maintaining both Ca^{2+} homeostasis and BDNF-mediated survival. Since even small increases in cytoplasmic Ca^{2+} can have deleterious consequences, including decreased neuronal survival (Johnson, et al., 1992), it is possible that the correction in the intracellular Ca^{2+} level by reduction of TrkB.T1 may contribute to the rescue of the Ts16 accelerated neuronal cell-death.

Taken together, our data provide evidence that imbalances in the physiological levels of TrkB receptor isoforms affect neuronal survival by altering both BDNF induced pro-survival signaling and Ca^{2+} homeostasis. Moreover, they suggest that the success of exogenous neurotrophin application to reduce or prevent cell death in neurodegenerative disorders may depend upon the neuron's ability to properly regulate the expression of neurotrophin receptor isoforms and, consequently, its survival.

Acknowledgements.

This work was supported by National Institutes of Health grants NR00174-03 (SGD) and R01NS40492 (BKK) and the National Cancer Institute, DHHS. We thank Mary Ellen Palko for providing assistance with the trkB.T1 targeting vector, Eileen Southon for ES cell manipulation, Susan Reid for blastocyst injection, Jodi Becker for animal handling and Vincenzo Coppola for BDNF quantitation in neuronal cultures.

Methods.

Generation of TrkB.T1 Mutant Mice. The targeting vector to conditionally remove the TrkB.T1 specific coding exon was constructed by a recombinogenic cloning strategy (Liu, et al., 2003). Briefly, a 10 kb sequence surrounding the T1 exon was retrieved from a C57bl/6j murine BAC clone (RP23 library, Invitrogen), into a pBluescript KS+ vector (Stratagene) containing a herpes virus thymidine kinase gene (HSV-TK) driven by the phospho-glycerate (pGK) promoter for negative selection. An upstream loxP site, containing a SpeI (S) restriction site, was placed 300 bp 5' of the T1 coding sequence. The pGK-neomycin (*NEO*) resistant cassette flanked by loxP and frt sites, used as a positive selection marker, was placed 1 kb 3' of the T1 coding sequence (Fig. 1). The targeting vector was electroporated in the CJ7 embryonic stem cell line (129/sv), and selection was performed as described (Tessarollo 2001). Genomic DNA was screened using a diagnostic SpeI digest with a 5' probe external to the targeting vector sequence (Figure 1A). Recombinant clones containing the expected 15 kb rearrangement were obtained at a frequency of 1/44. Two independent recombinant ES cell clones injected into C57BL/6 blastocysts produced chimeras that transmitted the targeted trkB.T1 allele to the progeny (Bonin et al. 2001). TrkB.T1 neo $^{+/-}$ mice were bred to β-actin CRE mice (Ma et al. 2003) to remove the NEO cassette and the TrkB T1 exon (Fig. 1a). Removal of the T1 exon, genomic DNA was verified by BamHI digest and use of probe B (Figure 1a, c). Breeding of two TrkB.T1+/– mice gave rise to homozygous mutants at a frequency of 25%.

Wheat Germ Immunoprecipitation and Western Blot Analysis. Cells were lysed in tris-buffered saline plus 0.01% Tween-20 (TBST) and protease/phosphatase inhibitors. Wheat germ agglutinin was added to the lysate and incubated for 4 hours at 4°C. The wheat germ pellet was washed and boiled for five minutes in 1x Sigma Sample Buffer (SSB, Sigma-Aldrich) for western blot analysis. For total protein extracts, cell lysates were SDS (Sigma)-solubilized, incubated at 100°C for 5 minutes, separated on a 4-12% NUPAGE bis-tris polyacrylamide gel (Invitrogen), and transferred to a nitrocellulose membrane. After blocking for 1-2 hours at room temperature in 5% non-fat dried milk (Carnation) in TBST, membranes were incubated for 8-12 hours with primary antibody at 4°C followed by incubation with the appropriate peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Sigma-Aldrich) in 5% TBST at

room temperature and visualized by chemiluminescence (Amersham Pharmacia Biotech). The rabbit polyclonal antibody directed to an extracellular epitope of trkB [$trkB_{out}$] was generously provided by Dr. Deborah Morrison (NCI-Frederick). The mouse monoclonal raised to the extracellular trkB epitope and the polyclonal antibody specific for phospho-trk were obtained from Cell Signaling. BDNF was from Upstate Biotechnology. Autoradiographs were scanned at 600 dpi using a Hewlett-Packard scanner and quantitation of band intensity was performed using NIH Image v1.62 (National Institutes of Health).

Generation of Trisomic Mice and Karyotyping. Female Rb(6:16)24Lub and male Rb(16:17)Bnr Robertsonian mice, singly heterozygous for the appropriate Robertsonian chromosome (Jackson Laboratory, Bar Harbor, ME) were mated to produce doubly heterozygous mice with both Robertsonian chromosome translocations [Rb(6:16)24LuB x Rb(16:17)7Bnr F1]. These doubly heterozygous Robertsonian mice were mated with wildtype or trkB.T1 homozygous C57BL/6J mice at generation N4 to generate wildtype and Ts16 mice heterozygous for trkB.T1. The next day was designated as day 0.5 of gestation (E 0.5). Normal and Ts16 fetuses were easily distinguishable; however, mice were also karyotyped by fluorescence in situ hybridization (FISH) using probes specific for chromosome(s) 6, 16 and 17 to confirm visual phenotyping. The TrkB.$T1^{+/-}$ mutation was verified by southern analysis as described in Fig 1.

Neuron and astrocyte cultures. Hippocampal neurons were dissected and cultured from euploid and Ts16 wildtype and trkB.T1 +/- E15.5 mice in minimal essential medium (MEM) and the serum-free supplement B27, as previously described (Bambrick, et al., 1995). Neurons were plated at 10^4 cells/cm^2 on 12 mm glass coverslips etched with a lettered grid (Eppendorf AG, Hamburg, Germany) for survival experiments and at 5 x 10^5 cells/35mm dish for Western blots. Glass coverslips were coated with poly-L-lysine (Sigma, St. Louis, MO) and mouse laminin. Plastic dishes were coated with poly-L-lysine alone. Unless otherwise indicated, all cell culture reagents were obtained from Invitrogen (Carlsbad, CA).

Astrocytes cultures were obtained from E15.5 embryos as described previously (Bambrick, et al., 1996). Briefly, cerebral cortices were separated from the meninges and the hippocampus removed. The cortices were placed in 2 ml of DMEM/F12 (DMEM/F12) with 10% fetal bovine serum (FBS, Hyclone) and penicillin G [50 U/ml] streptomycin [50 µg/ml] and

mechanically dissociated using a fire polished pasteur pipette. Approximately 400,000 cells/cm^2 were plated onto poly-L-lysine (Sigma-Aldrich) coated T25 tissue culture flasks. The medium was changed every three days in vitro (DIV), and by DIV 10, the flasks contained astrocytes that were 80-95% confluent. Glial fibrillary acidic protein (GFAP) immunostaining was conducted to verify that the majority (>90%) of cells were astrocytes (data not shown).

Measurement of Neuron Survival. At 2 days in vitro (div), all live neurons in each of four randomly selected 175 x 175 μm fields per coverslip (identified by the etched grid) and at least 8 coverslips per genotype (n=400+) were counted using phase-contrast microscopy. Cells with a pyknotic appearance were scored as dead, while cells with rounded, smooth somas and 2 or more projections were scored as live (Dorsey 2002). Neurons were counted every 24 hours, and survival was expressed as a percentage of cells present at 2 div that remained at 7 div. The significance of the difference between genotypes was determined by Student's t test.

Dye loading and Fluorescence measurement. Fura 2 acetoxymethylester (Fura 2AM, Molecular Probes, Inc., Oregon, USA) was suspended as a 1mM stock in Pluoronic DMSO. Isolated primary hippocampal neurons were then loaded at a fura 2AM concentration of 3 μm in Physiological Saline Solution (PSS; NaCL 140mM, KCl 4.0 mM, CaCl2 1.8 mM, KHrPO4 1.0 mM, MgCl 1.4 mM, Hepes 10.0 mM, Glucose 11.5 mM) for 40 min. Cells were imaged after two washes for 10 min in PSS, and resting for 30 min. Relative intracellular $[Ca^{2+}]_{free}$ was determined by means of a ratiometric fluorescence method reported previously (Chun *et al.*, 2003). Coverslips were imaged on an inverted microscope (Olym. IX-50; 40x H_20 Obj.). Fields of cells were excited alternately at 340nm and 380 nm (Sutter DG-4; Chroma filter set) while the emission at 515nm was collected (CCD, Roper CoolSnap HQ). On archived image sequences (5 samples/field at 0.2 Hz) cell boundaries were manually determined and the background corrected fluorescence emission ratio (i.e., $340_{ex}/380_{ex}$) was determined for each data point. The mean fluorescence ratio was calculated for each sequence and used as the relative $[Ca^{2+}]_{free}$ value for the individual cell. System automation and data analysis was performed with IPlab 4.1 (Scanaltytics, Fairfax Va. USA). Intracellular calcium measurements were compared using non-parametric analysis (Mann-Whitney Test on Ranks) with significance set at $p < 0.05$.

Figure Legends.

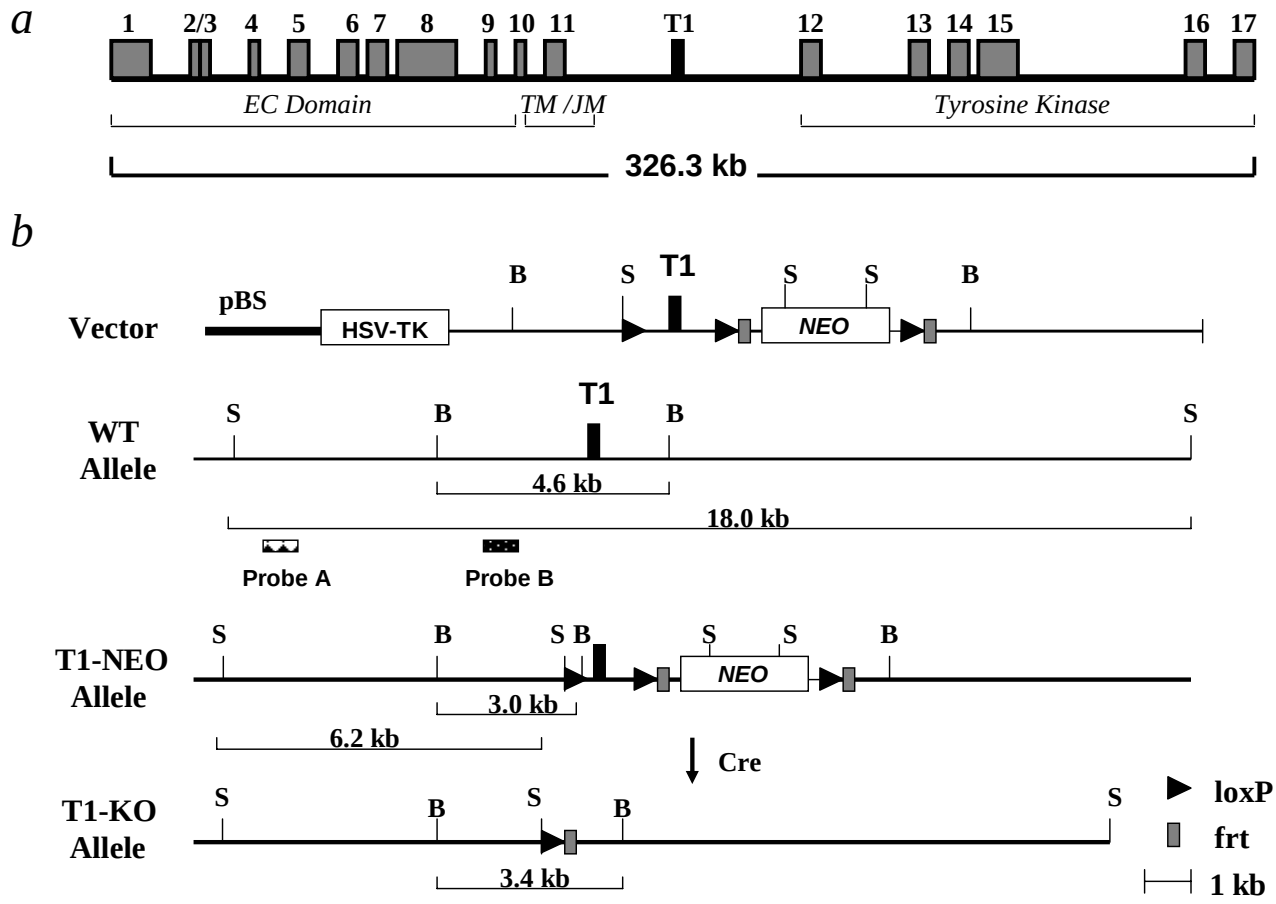
Figure 1. TrkB.T1 deletion does not affect full-length TrkB expression. (a) Diagram of the TrkB locus showing the position of the TrkB.T1-specific coding exon relative to the exons encoding the tyrosine kinase catalytic domain. (b) Schematic representation of the replacement type vector and strategy used to delete the trkB.T1 exon. Restriction enzyme sites are as indicated. B, *Bam*HI; S, *Spe*I. pBS indicates the pBluescript cloning vector. Southern blot analysis of ES cell DNA using an *Spe*I digest and probe A detected the rearrangement in the mouse trkB locus (not shown). (c) Southern blot analysis of tail DNA from a litter obtained by intercrossing a trkB.T1-*NEO* $^{+/-}$ mouse with a β-actin CRE $^{+/+}$ mouse, using a BamHI digest and probe B shown in panel a. Note the switch of the 3 Kb band (center lane) to 3.4 Kb (right lane) after Cre excision of the TrkB.T1 exon. (d) Specific loss of the TrkB.T1 isoform in whole brain lysates prepared from trkB.T1$^{-/-}$ animals. Western blot analysis using the $trkB_{out}$, antibody as described in methods (left panel). The bottom panel shows the blot stripped and re-probed with an antibody to actin to demonstrate protein loading. The right panel represents a wheat germ agglutin precipitation from whole brain lysate, probed with $trkB_{out}$. Note the specific loss of the truncated TrkB receptor. (e) Targeting of TrkB.T1 does not affect expression of the TrkB kinase receptor in primary astrocyte cultures. Astrocytes were prepared from trkB.T1 +/+, +/– and –/– mice, lysed and analyzed by western blot using the $trkB_{out}$ antibody (top panel). The bottom panel shows an immunoblot for actin as a loading control.

Figure 2. Reduction of TrkB.T1 levels in the Ts16 mouse restores hippocampal neuron survival and decreases dendritic fragmentation. (a) Deletion of one *TrkB.T1* allele restores the physiological level of TrkB.T1 in Ts16 hippocampal neurons. Neuronal cultures were prepared from embryonic euploid and Ts16 littermates that were either wildtype for trkB.T1 or heterozygous for trkB.T1. Lysates from neuronal culture from different genotypes were prepared and analyzed by western blot using an antibody directed to the extracellular domain of TrkB. The autoradiographs shown are representative of three independent experiments. (b) Cultured hippocampal neurons prepared from E15.5 embryos were assayed for survival over seven days in vitro as described in Methods. Approximately 400 neurons for each genotype were analyzed for survival, expressed as a percentage of the number of cells at day two *in vitro*. Error bars show

S.E. and the number of embryos analyzed for each genotype is indicated in parenthesis. (c) Phase contrast images of euploid, Ts16 and Ts16-TrkB.T1$^{+/-}$ cultured hippocampal neurons at six days *in vitro*. Note the extensive fragmented processes and cellular debris in the Ts16 but not in the TrkB.T1$^{+/-}$ cultures. Bars indicate 20 μm.

Figure 3. Reduction of TrkB.T1 in Ts16 neurons restores BDNF-induced TrkB activation and resting cytoplasmic Ca^{2+} levels. (a) E15.5 hippocampal neuron cultures of the indicated genotypes were cultured with B27 serum supplement. On the third day *in vitro*, the supplement was removed for four hours, and neurons were either left untreated as controls (not shown), or treated for five minutes with 100 ng/ml of BDNF. Lysates were harvested an analyzed by western blot using an antibody specific for trk phosphorylation at the Y490 site. The autoradiograph shown is representative of three independent sets generated from the various genotypes. (b) Quantification of TrkB phosphorylation. TrkB activation in euploid neurons was set at 100%. Ts16 and TrkB.T1$^{+/-}$ neuron activation is expressed as % of euploid neuron activation. Error bars indicate S.E. (n=3). * $p < 0.01$ by t-test. (c) Cytoplasmic Ca^{++} is reduced in hippocampal neurons from Ts16-TrkB.T1$^{+/-}$ mice. Box plots indicate the 25th, 50th (median), and 75th percentiles of the distribution of fura 2 fluorescence emission ratios from at least 36 neurons each of the indicated genotypes. Whisker lines indicate the range from 1 to 99% of the distribution. All groups are significantly different from one another at $p < 0.05$.

Figure 1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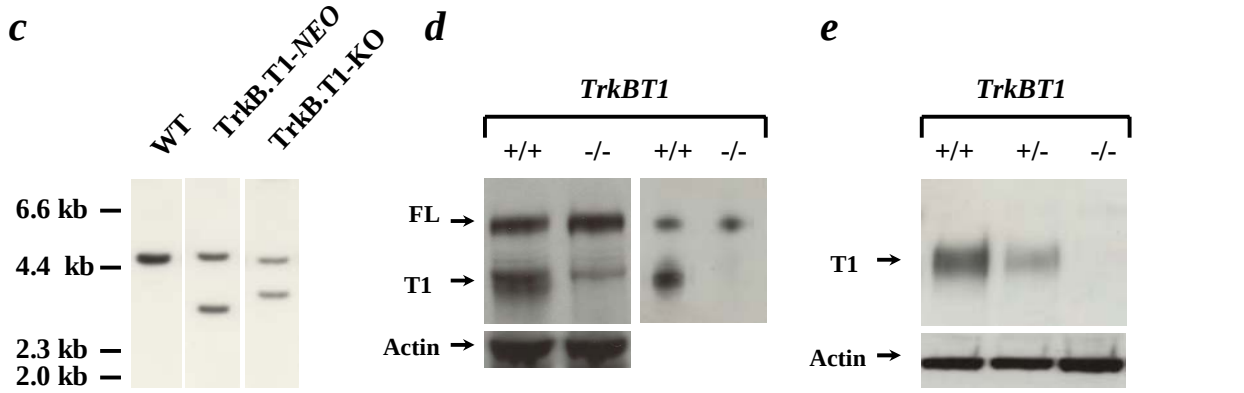
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Figure 2

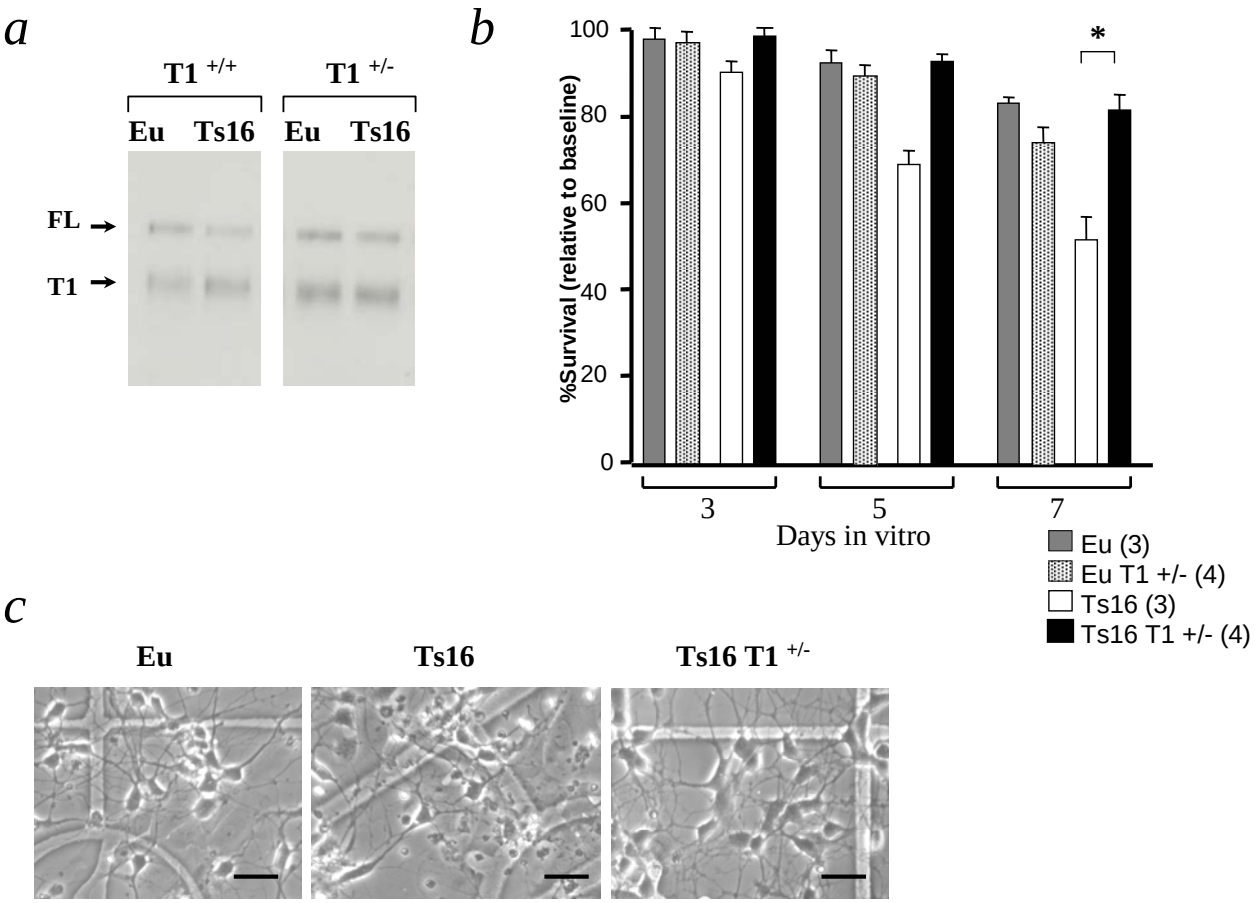
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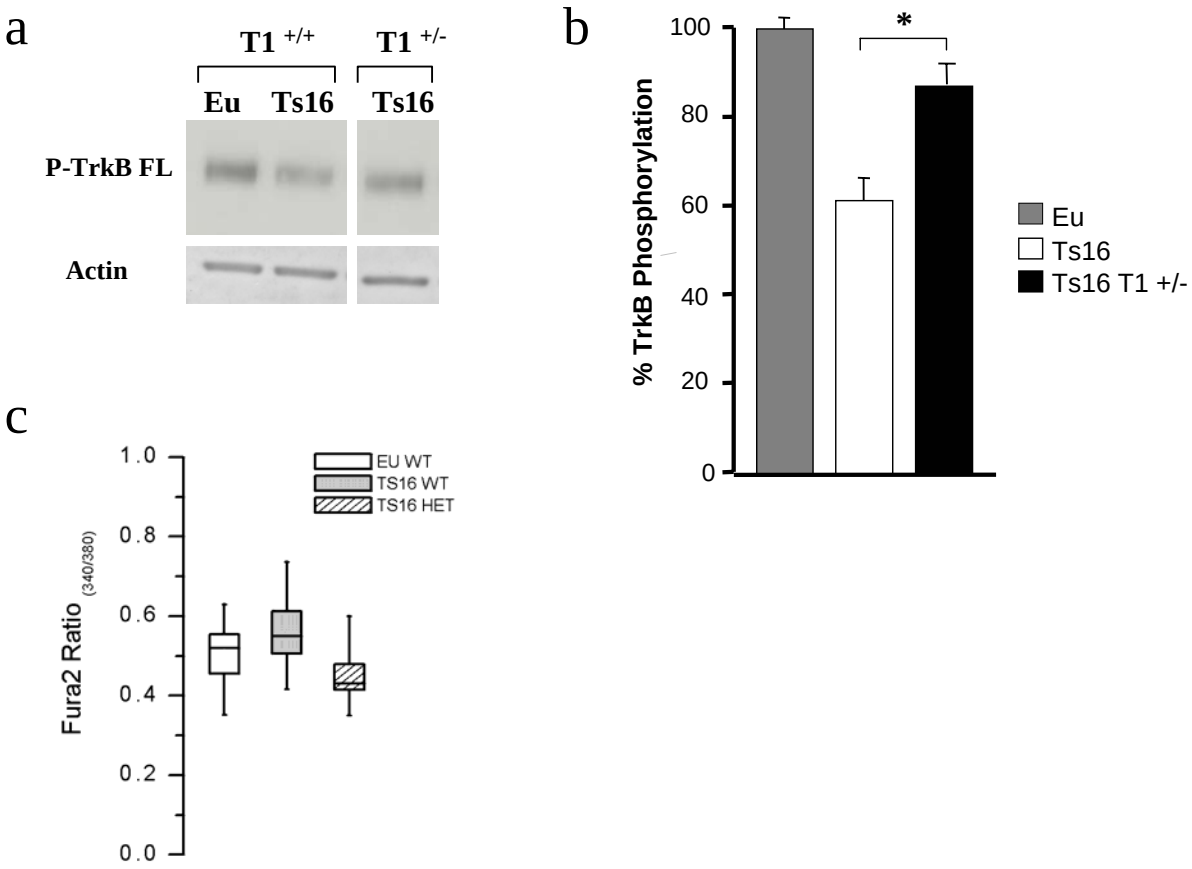
Figure 3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